Absence of Cutaneous TNFα-Producing CD4$^+$ T Cells and TNFα may Allow for Fibrosis Rather than Epithelial Cytotoxicity in Murine Sclerodermatous Graft-Versus-Host Disease, a Model for Human Scleroderma

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Graft-versus-host disease (GVHD) is a complication of hematopoietic cell transplantation and is a major source of morbidity and mortality. Two main forms of GVHD occur: cytotoxic GVHD (Cyt GVHD), in which TNFα is a critical cytokine in epithelial injury, and sclerodermatous GVHD (Scl GVHD), in which TGFβ plays a major role in fibrosis. To understand the critical early events in GVHD and scleroderma, we are studying a murine model that uses differences in minor histocompatability antigens to generate Scl GVHD. We asked the question: what is the immune environment in this model that promotes fibrosis rather than the epithelial injury of Cyt GVHD? We found that in Scl GVHD, cutaneous CD4$^+$ T cells produced IFNγ and IL-2 but not TNFα, also absent by gene array analysis. The role of cutaneous CD4$^+$ T cells in Scl GVHD may not be an active process through production of TGFβ, but may rather be a passive one due to lack of antigen-presenting cell (APC) support for CD4$^+$ T cells and failure to produce TNFα, a potent inhibitor of TGFβ-induced fibrosis as well as inducer of keratinocyte apoptosis. These APC–T cell interactions and the cytokine environment promote fibrosis rather than cytotoxic epithelial injury in skin in Scl GVHD.

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INTRODUCTION

Graft-versus-host disease (GVHD) is the major complication of hematopoietic cell transplantation with high morbidity and mortality. There are two major forms of GVHD, acute cytotoxic or chronic GVHD. Cytotoxic GVHD (Cyt GVHD) is characterized by epithelial injury. When it progresses to chronic disease, it is typically a lichenoid or lupus-like chronic interface dermatitis in skin. A subset of chronic GVHD is sclerodermatous GVHD (Scl GVHD), which is scleroderma-like and tends to be more chronic and fibrotic with minimal epithelial injury. For long-term survivors of hematopoietic cell transplantation there is a 40–60% chance of developing chronic GVHD. Occurrence, severity, and form of GVHD depend on the degree of human leukocyte antigen mismatch and on the host conditioning [X-ray therapy and chemotherapy to ablate recipient bone marrow (BM)] before transplantation. Even when human leukocyte antigen-identical donor cells are used, minor histocompatability antigen mismatches can still trigger GVHD (Vogelsang *et al.*, 2003).

Cyt GVHD is thought to occur in three stages, which consist of a conditioning phase (cytokine storm), donor T-cell activation phase, and effector phase in which cytotoxic T cells, natural killer cells, and inflammatory cytokines cause epithelial cell injury (dermatitis, diarrhea, and hepatitis in that order) (Ferrara *et al.*, 1996, 1999; Vogelsang *et al.*, 2003). Tumor necrosis factor (TNF)α has a critical role in Cyt GVHD as it is released in response to lipopolysaccharide from gut flora entering the circulation through damaged gastrointestinal epithelium and is part of the ''cytokine storm'' that can activate resident antigen-presenting cells (APCs). TNFα is also part of the effector phase because it can induce apoptosis, a major manifestation of epithelial injury. Blocking TNFα with antibodies is effective in steroid-resistant Cyt GVHD (Couriel *et al.*, 2004).

The fibrosing variant of GHVD, Scl GVHD, makes up approximately 10–15% of chronic GVHD (Vogelsang *et al.*, 2003) and is less well understood. Like scleroderma, individuals with Scl GVHD develop skin and lung fibrosis and disease is typically refractory to immunosuppressant treatment. The cytokine environment leading to fibrosis in Scl

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Abbreviations: Cyt, cytotoxic; DC, dendritic cell; GVHD, graft-versus-host disease; Scl, sclerodermatous

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GVHD has not been studied, but TGFβ is known to be a major driver of fibrosis in scleroderma. Well-established scleroderma is thought to have a Th2-like cutaneous cytokine environment (Abraham and Varga, 2005).

To understand the mechanisms that initiate Scl GVHD, we examined early events in a murine Scl GVHD model generated by transplanting B10.D2 (H-2^d) BM and spleen cells into lethally irradiated Balb/c mice (H-2^d). Balb/c mice transplanted with syngeneic Balb/c BM and spleen cells serve as controls [control BM transplantation (BMT)]. We have previously shown that CD11b$^+$ macrophages predominate over CD3$^+$ T cells in skin in early Scl GVHD. The cutaneous macrophages express scavenger receptors, suggesting a role in antigen presentation (McCormick *et al.*, 1999; Zhang *et al.*, 2003). Messenger RNA (mRNA) for TGFβ1 is upregulated by approximately four- to sixfold by RT-PCR (McCormick *et al.*, 1999; Zhang *et al.*, 2003) and by gene array analysis (Zhou *et al.*, 2007) and monocyte/macrophages are the major producers of TGFβ in skin (Zhang *et al.*, 2002a).

Because the earliest initiating events of self-reactivity would be expected to occur in the spleen in transplanted animals, we have focused on the APC–T cell interactions and the cytokine environments in spleen and skin and asked these questions: (1) what are the major effector T cells and APCs that activate them in Scl GVHD? (2) What is the immune environment that leads to TGFβ-driven fibrosis rather than TNFα-driven cytotoxicity?

Here, we demonstrate that CD4$^+$ T cells are activated in spleen in early murine Scl GVHD, whereas only CD11c$^+$ dendritic cells (DCs) are activated in the spleen. Activated CD4$^+$ T cells are found in the skin of normal mice, control BMT mice, and in Scl GVHD mice, whereas CD11b$^+$ macrophages and CD11c$^+$ DCs are activated in the skin only in Scl GVHD mice. Therefore, there are different APC–T cell interactions in spleen and skin in Scl GVHD mice and the presence of activated T cells by itself is not sufficient to activate resident APCs in the skin.

Splenic CD4$^+$ T cells make TNFα, but cutaneous CD4$^+$ T cells do not, and TNFα mRNA is not present by gene array analysis, an important finding in explaining the lack of epithelial cell injury in Scl GVHD. Thus, although splenic CD11c$^+$ DCs are associated with TNFα production, neither cutaneous CD11b$^+$ macrophages nor CD11c$^+$ DCs are associated with TNFα production. Thus, TNFα production by CD4$^+$ T cells may be dependent on the local APC–T cell interactions. The failure of CD4$^+$ T cells to make TNFα may account for the development of Scl GVHD instead of Cyt GVHD, because TNFα is a potent inhibitor of TGFβ-induced fibrosis as well as a critical factor in keratinocyte apoptosis.

RESULTS

Increased skin thickening and not epithelial or follicular keratinocyte apoptosis is a hallmark of Scl GVHD

We examined hematoxylin and eosin staining of ear and skin sections of either normal mice or mice at 21 days post-BMT. Ear sections from normal mice (not irradiated or transplanted) and control BMT mice showed little evidence of increased cellular infiltration or thickening of the dermis (Figure 1a, upper panel). In contrast, increased cellular infiltration and dermal thickening are seen in ears and skin of mice when B10.D2 BM and spleen cells are transplanted (Scl GVHD). During this early stage we find essentially normal epidermis in Scl GVHD mice, where apoptotic keratinocytes can rarely be found (Figure 1b, lower panel). This is in contrast to Cyt GVHD, which results when C57BL/6 BM and spleen are transplanted to Balb/c mice. The diagnostic feature of Cyt GVHD is the formation of many apoptotic keratinocytes (white arrows, Figure 1b, lower panel) in epidermis and hair follicles (''epithelial injury out of proportion to the inflammation'') (Gilliam *et al.*, 1996; Yoo *et al.*, 1997). Examination of gut histology of Scl GVHD also showed no GVHD-induced apoptosis (data not shown). This is consistent with absence of diarrhea in Scl GVHD mice.

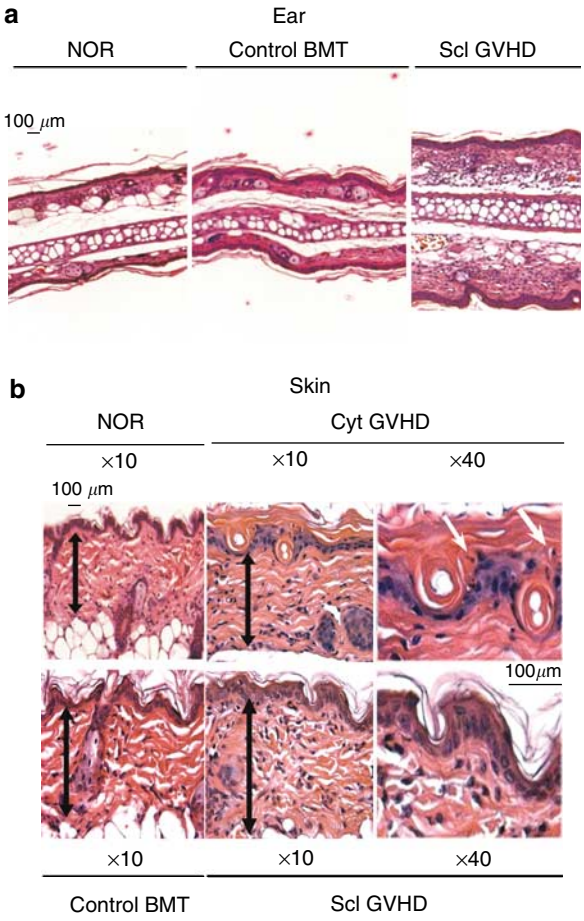
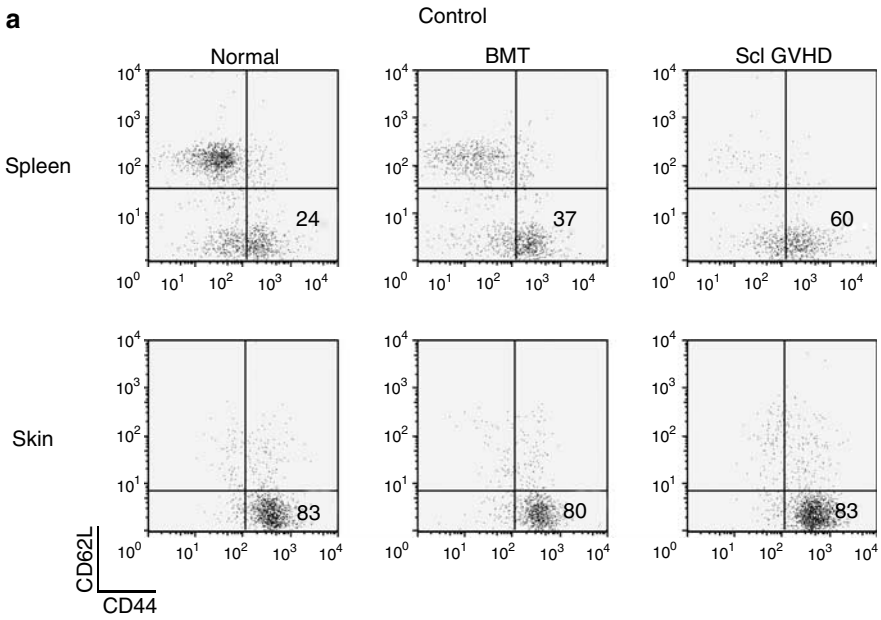


Figure 1. Skin thickening and not keratinocyte apoptosis is characteristic of Scl GVHD. Tissue was harvested from either (**a**) ear or (**b**) skin and examined after hematoxylin and eosin staining of paraffin-embedded tissue. Representative histology of one mouse in each group of three to five mice is shown. Both cellular infiltration and skin thickening (black arrows), but no epithelial injury is evident 21 days post-BMT in Scl GVHD mice. Inflammation and skin thickening are not seen in normal (NOR) or control BMT mice. There is no skin thickening in mice with Cyt GVHD (C57BL/6→Balb/c) but cellular infiltration and epithelial injury (white arrows identify apoptotic keratinocytes) occurs. Black bar represents 100 μm.

Activated CD4 T cells predominate in early Scl GVHD in both spleen and skin, but APC activation depends on tissue site

To ensure the development of GVHD in our model, we added spleen cells that contain mature T cells that will presumably react with recipient APCs, be activated, and cause GVHD. Therefore, we evaluated activation rather than proliferation of transplanted T cells, as determined by the change of expression from $CD62L^+CD44^-$ (naïve T cells) to $CD62L^-CD44^+$ (activated T cells). We used flow cytometry analysis of single-cell suspensions of spleens and skin from either normal mice or mice after 7 or 14 days post-BMT in either control BMT (Balb/c→Balb/c) or experimental Scl GVHD (B10.D2→Balb/c) groups. Most splenic $CD4^+$ T cells are naïve ($CD44^-CD62L^+$) in both normal (unirradiated, not transplanted) and control BMT mice, but become activated ($CD44^+CD62L^-$) in mice developing Scl GVHD by day 7 post-BMT (Figure 2a). Percentage of $CD44^+CD62L^-$ cells in spleen of normal and control BMT mice is 24 and 37%, respectively, compared with 60% in Scl GVHD. In data not presented here, most splenic $CD8^+$ T cells were nonspecifically activated as we found equal percentage of $CD44^+CD62L^-$ cells in both BMT control mice and mice with Scl GVHD, suggesting that $CD8^+$ T cell activation is not dependent on GVHD in this model. The percentage of activated $CD4^+$ T cells in the spleen decreased over time, suggesting migration of activated T cells out of spleen, apoptosis of activated T cells, and/or replacement by newly synthesized T cells from donor BM cells (data not shown). When we calculated exact number of naïve or activated $CD4^+$ T cells in the spleen, as expected we found more T cells in the normal mice compared with control or Scl GVHD mice, but the ratio of naïve to activated was similar in normal and control group (2:1 in normal and 3:1 in control, Figure 2b). There was a tremendous skewing in the number of naïve $CD4^+$ T cells compared with activated during Scl GVHD (1:7 ratio of naïve to activated T cell, Figure 2b). The numbers of T cells increase over time post-BMT in both control and Scl GVHD because of the repopulation of the spleen from T cells generated from the BM. We found that in mice given BM alone (either Balb/c or B10.D2), $CD4^+$ T cell numbers do not start to increase until after day 21 post-BMT.



b

Tissue	Treatment	Naïve ($CD62L^+CD44$) ($\times10^{-4}$)	Activated ($CD62L^-$ $CD44^+$) ($\times10^{-4}$)	Ratio naïve $CD4^+$:activated $CD4^+$
Spleen ($CELL^{\#}$/ SPLEEN)	NOR	410	180	2:1
	CON	26	7.4	3:1
	Scl GVHD	12	89	1:7
Skin ($CELL^{\#}$/ g SKIN)	NOR	0.95	20	1:21
	CON	1.3	21	1:16
	Scl GVHD	2.5	42	1:17

Figure 2. Activation of T cells in spleen and skin in Scl GVHD. $CD4^+$ T cells were examined by flow cytometry for expression of activation markers 7 days after BMT. (**a**) Activated $CD4^+$ T cells were evaluated by using single-cell suspensions pooled from 3–5 mice/group of either spleen or skin stained with anti-CD4 antibodies in combination with antibodies for activation markers CD44 and CD62L. The percentages of $CD4^+$ T cells that are activated ($CD44^{hi}CD62L^{neg}$) are indicated in the right lower quadrant. Although activated T cells in the spleen are increased in Scl GVHD mice, most cutaneous $CD4^+$ T cells are activated in all three groups. (**b**) Absolute numbers (#) of naïve ($CD44^-CD62L^+$) or activated ($CD44^+CD62L^-$) in spleen or skin of normal, control BMT mice, or Scl GVHD mice were determined by flow cytometric analysis of single-cell suspensions.

In single-cell suspensions from skin, all $CD4^+$ T cells were $CD44^{hi}CD62L^{neg}$ whether isolated from normal (83%), control BMT (80%), or Scl GVHD (83%) mice (Figure 2a) suggesting that only activated T cells are found in the cutaneous environment. This would be expected, as $CD62L^+$ naïve cells would not migrate through peripheral tissue. When we examined absolute number of $CD4^+$ T cells isolated from skin, we found that both normal and control mice showed equivalent numbers of naïve and activated T cells (Figure 2b), whereas Scl GVHD showed almost twofold increase in both naïve and activated T cells. Despite the differences in cell number, the ratio of naïve to activated $CD4^+$ T cells was consistent (1:21 for normal, 1:16 for control BMT, and 1:17 for Scl GVHD).

To examine APC activation in the spleen and skin, we focused on the B7 family of costimulatory molecules (CD86 and PDL1/B7H1) because of their essential role in initiating and regulating T-cell response (Rothstein and Sayegh, 2003). In control BMT mouse spleen at day 7 post-BMT, only $CD11c^+$ DCs constitutively expressed CD86 (specific mean fluorescent value = 64), which is an essential protein in activating T cells. Splenic $CD11c^+$ DCs from Scl GVHD mice (thick-line plot), compared with control BMT (thin-line plot) showed activation as determined by increased expression of CD86 and PDL1/B7-H1 (Figure 3, upper panel). The numbers in regular and bold font show the specific mean fluorescent value for control BMT and Scl GVHD, respectively. For splenic $CD11c^+$ DCs, the specific mean fluorescent value increases from 64 to 90 for CD86 expression and from 60 to 99 for PDL1. This activation is diminished by 21 days post-BMT (data not shown). Splenic $CD11b^+$ macrophages expressed very low levels of CD86 and PDL1/B7-H1, which were not influenced by Scl GVHD, as there is little difference for control BMT and Scl GVHD (Figure 3, upper panel). In skin, both $CD11b^+$ macrophages and $CD11c^+$ DCs constitutively express CD86 and PDL1/B7-H1. They also show increased expression of CD86 and PDL1/B7-H1 during Scl GVHD, shown by the shift in specific mean fluorescent value in Scl GVHD compared with control BMT (Figure 3, lower panel). This suggests that unlike in spleen, cutaneous $CD11b^+$ macrophages and $CD11c^+$ DCs are being activated. In addition, cutaneous APCs would not be signaling to the T cells predominately through CD28 (ligand for CD86), but may be using PD-1 (ligand for PDL1) instead. This interaction is known to promote T-cell apoptosis and IL-10 production (Dong *et al*., 1999, 2002). This is an important observation suggesting that cutaneous APCs would not be supportive of T-cell proliferation or cytokine production.

Cutaneous APCs fail to support activated $CD4^+$ T cells *in vitro* in the Scl GVHD model, but not the Cyt GVHD model

To evaluate the ability of cutaneous APCs to support the ability of activated $CD4^+$ T cells that would be entering the skin during Scl GVHD, we tested cutaneous $CD11b^+$ macrophages and $CD11c^+$ DCs for their ability to support the proliferation of B10.D2 $CD4^+$ T cells that had previously been activated by splenic $CD11c^+$ DCs. To determine if the APC–T cell interaction was dependent on APC population or population of $CD4^+$ T cell encountered, we also examined the ability of cutaneous $CD11b^+$ and $CD11c^+$ cells in supporting proliferation of C57BL/6 $CD4^+$ T cells, a population of T cells that can generate Cyt GVHD. Although $CD11b^+$ macrophages and $CD11c^+$ DCs were not supportive of B10.D2 $CD4^+$ T-cell proliferation (Figure 4a), both populations of cutaneous APCs were supportive of C57BL/6 $CD4^+$ T cell proliferation (Figure 4b).

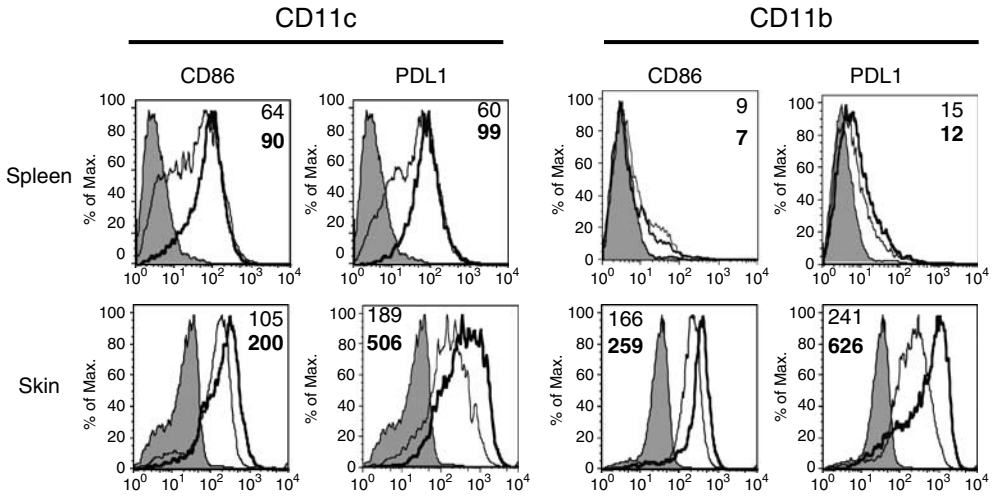


Figure 3. Activation of APCs in spleen and skin in Scl GVHD. Purified $CD11c^+$ DCs and $CD11b^+$ macrophages from spleen or skin were stained with either anti-CD86 or -PDL1 antibodies and examined by flow cytometry for costimulatory molecules that would be important in T-cell activation. In the spleen, only $CD11c^+$ DCs were activated based on increased expression of CD86 and PDL1, whereas in the skin, both $CD11c^+$ and $CD11b^+$ cells showed increased expression of PDL1, an inhibitory rather than stimulatory molecule. Numbers in normal font represent specific mean fluorescent value of control BMT, whereas numbers in bold font represent specific mean fluorescent value from Scl GVHD (Isotype control Ab – gray-filled plot; control BMT – thin-lined plot; Scl GVHD – thick-lined plot). Tissues from 3 to 5 animals were pooled together for analysis. The results are representative of four separate experiments.

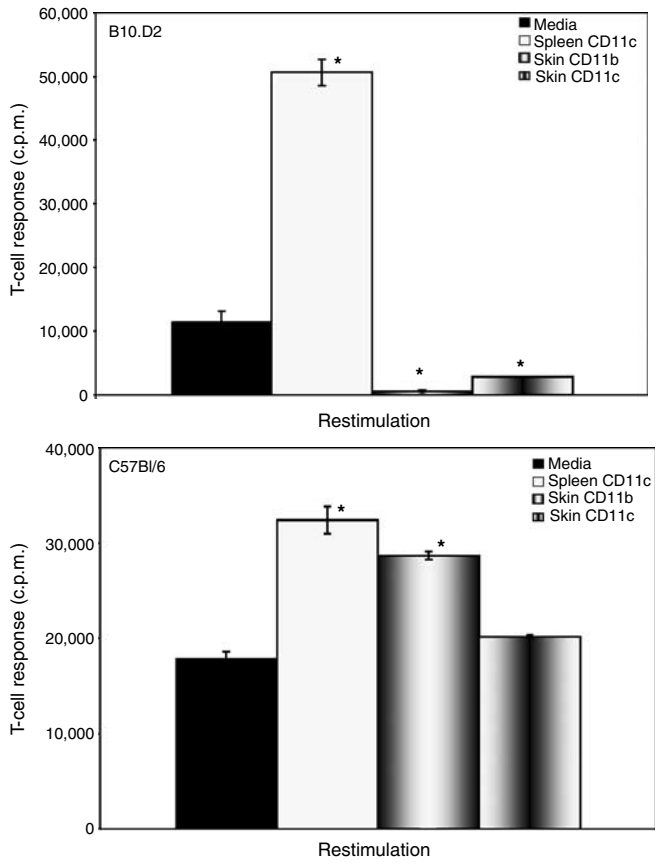


Figure 4. Capacity of cutaneous APCs to function as stimulators of activated CD4$^+$ T cells in mixed leukocyte reactions depends on specific T-cell population. Cutaneous APCs were examined for their ability to restimulate activated CD4$^+$ T cells from either (**a**) B10.D2 or (**b**) C57Bl/6 mice. Although neither CD11b$^+$ nor CD11c$^+$ cutaneous APCs were capable of restimulating activated B10.D2 CD4$^+$ T cells, both populations could restimulate C57Bl/6 CD4$^+$ T cells. Values identified by * were found to be significantly different ($P<0.01$) from media-alone controls. Results are representative of three separate experiments.

What is the T-cell cytokine profile in spleen and skin in early Scl GVHD?

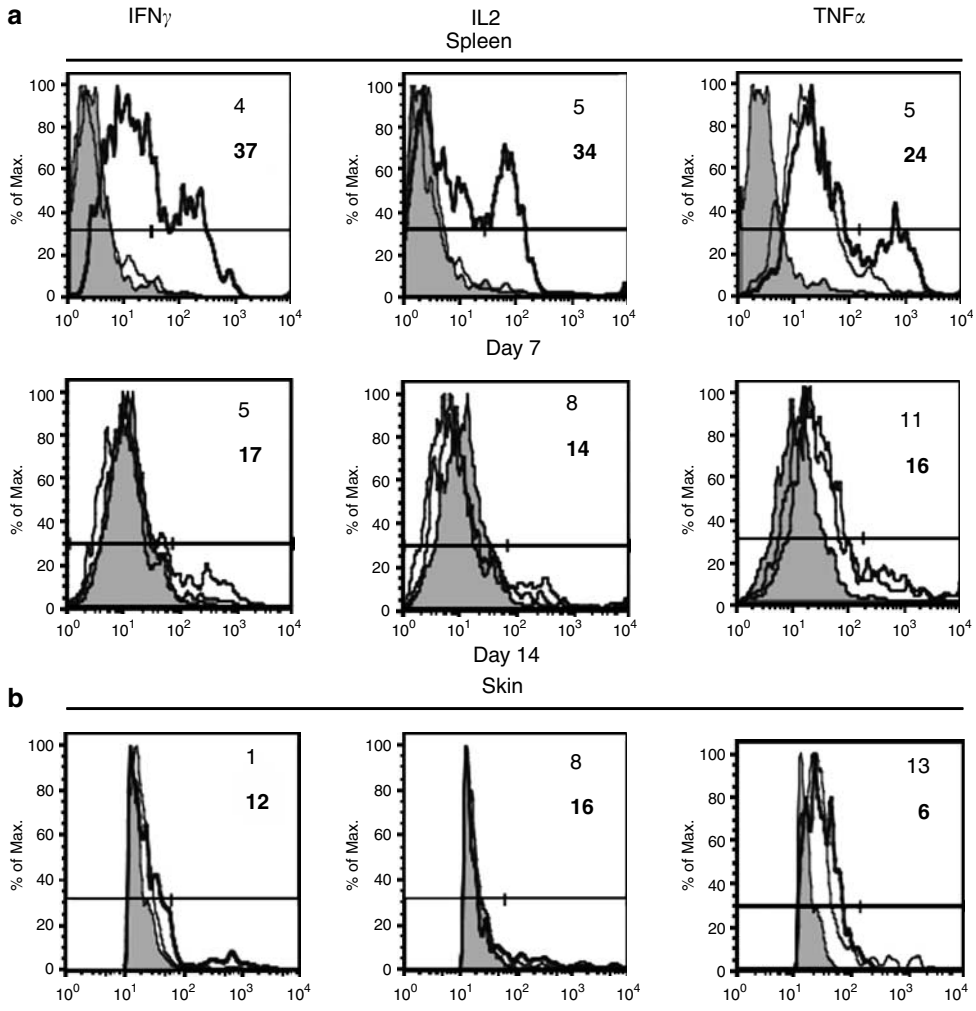
We examined the ability of CD4$^+$ T cells from spleen and skin to synthesize cytokines in short-term culture. Numbers of CD4$^+$ T cells synthesizing IFNγ, IL-2, and TNFα were evaluated by flow cytometric analysis (Figure 5). Splenic CD4$^+$ T cells from mice developing Scl GVHD producing IFNγ, IL-2, and TNFα are increased by day 7 post-BMT (thick-line plot, Figure 5, upper panels) compared with control BMT mice (thin-line plot). This production of cytokines in the spleen decreased over time with few cells producing cytokines by day 14 and day 21 post-BMT, correlating with decrease in activated T cells in the spleen over time (Figure 5, middle panels; day 21 not shown). Activated cutaneous CD4$^+$ T cells are found in the skin of all mice, but only CD4$^+$ T cells isolated from skin of mice with Scl GVHD produced IFNγ and IL-2 (Figure 5b). Unlike their splenic counterparts, few cutaneous CD4$^+$ T cells produced TNFα. The data for skin cytokine-producing cells are presented in the chart at the bottom of Figure 5.

We used gene array analysis of total skin mRNA from normal mice, control BMT mice, and Scl GVHD mice to evaluate the cutaneous cytokine mRNA environment in early Scl GVHD (day 30). We found that skin from Scl GVHD mice had increased mRNA expression for the following cytokines: IFNγ, IL-2, IL-10, IL-18, and TGFβ1 (Figure 6). No IFNγ or IL-2 mRNA expression was seen in control BMT mice, and we found lower levels of IL-10, IL-18 and TGFβ1 in control BMT mice compared with Scl GVHD mice. IL-10 mRNA expression in the skin of mice undergoing Scl GVHD is increased compared with control BMT (156 *vs* 30, fivefold increase). We failed to identify any IL-10-producing CD4$^+$ T cells by flow cytometry (data not shown), suggesting that other cells such as macrophages may be expressing IL-10 mRNA. IL-18 was increased in Scl GVHD compared with control BMT mice (1,819 *vs* 574, threefold increase). One major function of IL-18 is to enhance IFNγ production (Nakanishi *et al.*, 2001; Tsutsui *et al.*, 2004). IL-18 may help to maintain a strong Th1-like environment. The fold increase in TGFβ1 (Scl GVHD – 408 *vs* control BMT – 83, fivefold increase) is consistent with our previously reported results by semiquantitative RT-PCR (Zhang *et al.*, 2002a). In either Scl GVHD or control BMT groups, there was no expression of IL-4, IL-5, or TNFα (Figure 6). The cytokine environment (IFNγ, IL-2, IL-18 high, no IL-4, or IL-5) suggests infiltration by Th1-like T cells in early fibrosing disease. In addition, mRNA for TNFα, a cytokine critical to epithelial cell injury, is not present in the skin of mice undergoing Scl GVHD. These data have been confirmed by RT-PCR analysis and are reported separately (Zhou *et al.*, 2007).

DISCUSSION

Pathophysiology of Scl GVHD

The mechanism for GVHD initiation is dependent on the model studied. Cyt GVHD is characterized by epithelial injury and is mediated by CD4$^+$ and CD8$^+$ T cells and natural killer cells, with cytokines such as TNFα and IFNγ critical for disease (Shustov *et al.*, 1998; Kataoka *et al.*, 2001; Ichiki *et al.*, 2006). For instance, C57BL/6 donor (H-2^b) cells transplanted into lethally irradiated Balb/c mice (H-2^d) will produce Cyt GVHD as shown in Figure 1. In Scl GVHD, CD4$^+$ T cells and macrophages are the major cells involved (McCormick *et al.*, 1999; Zhang *et al.*, 2002a, 2003; Kaplan *et al.*, 2004; Anderson *et al.*, 2005). Figure 1 also shows that B10.D2 donor (H-2^d) cells produce Scl GVHD in the same recipient mouse strain used to generate Cyt GVHD using C57BL/6 donors (McCormick *et al.*, 1999; Zhang *et al.*, 2002a; Zhang *et al.*, 2003). Although the C57BL/6→Balb/c mismatch is across major histocompatability loci, transplantation across minor histocompatability loci can also produce Cyt GVHD. Others have shown that using congenic mice that differed at major histocompatibility complex loci it is possible to generate either cytotoxic or Scl GVHD (Kaplan *et al.*, 2004). Therefore the form of GVHD may be dependent on the target antigens selected by the different major histocompatibility complex molecules based on binding affinity of peptides to the major histocompatibility complex molecules. The combination of different peptides and different major



Day		IFNγ	IL2	TNFα
7	CON BMT	1	8	13
	Scl GVHD	12	16	6
14	CON BMT	1	3	20
	Scl GVHD	9	6	10

Figure 5. Splenic and cutaneous CD4$^+$ T cells differ at production of TNFα in Scl GVHD. (**a**) Splenic CD4$^+$ T cells isolated 7 days after BMT from Scl GVHD mice (thick-line plot) showed increased number of IFNγ-, IL-2-, and TNFα-producing CD4$^+$ T cells compared with control BMT mice (thin-line plot) and isotype control (gray-filled plot). The percentage of CD4$^+$ T cells producing cytokines decreased by day 14 post-BMT. (**b**) Scl GVHD mice showed increased cutaneous CD4$^+$ T cells expressing IFNγ and IL-2, but not TNFα-positive cells compared with control BMT mice 7 days after BMT. We also found fewer IFNγ- and IL-2-producing CD4$^+$ T cells 14 days post-BMT. Tissues from five animals were pooled together for analysis. The results are representative of three separate experiments.

histocompatibility complex molecules will allow selection for different T-cell repertoires that may account for the GVHD phenotype (Kaplan *et al.*, 2004).

Where and how is GVHD initiated?

Splenic DCs are sufficient to induce GVHD by activating donor T cells that are different at major histocompatibility complex or minor histocompatability complex loci (Duffner *et al.*, 2004). In a study to examine the role of resident APCs in promoting infiltration of GVHD-causing T cells into different tissues, mice that had splenic and hepatic APCs depleted by liposomes were injected with splenic T cells isolated from mice undergoing Cyt GVHD (Zhang *et al.*, 2002b). Although these mice showed no evidence of GVHD in the spleen or liver, they developed cutaneous GVHD, suggesting that activation of T cells in the spleen results in

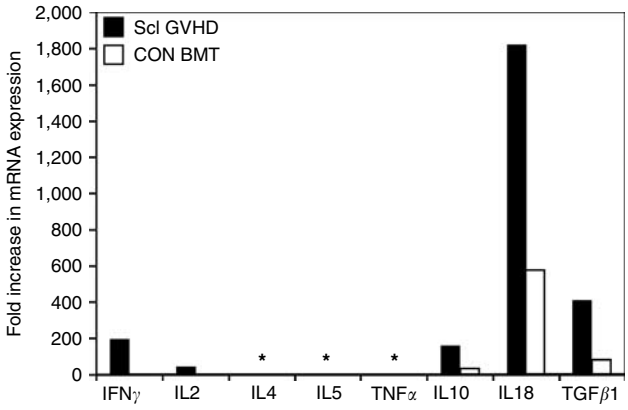


Figure 6. Induction of mRNAs for cytokine genes in Scl GVHD mice. Skins from normal mice, control BMT mice (Balb/c→Balb/c), and Scl GVHD mice (B10.D2→Balb/c) were isolated and RNA was extracted from whole tissue at day 30. Gene expression in Scl GVHD mice (closed bar) and control BMT mice (open bar) were compared with mRNA in normal mice. There was no detectable mRNA for TNFα, IL-4, or IL-5 in skin in either Scl GVHD mice or control BMT mice as indicated by * in the graph. The results are representative of three separate experiments.

generation of GVHD-causing skin-homing T cells. Expression of CD80/CD86 by splenic APCs is important in inducing cutaneous GVHD (Anderson *et al.*, 2005). In our studies, we found that only splenic CD11c$^+$ DCs constitutively express CD86, suggesting that they are capable of initiating GVHD by activating T cells. In addition, splenic CD11c$^+$ DCs also show upregulation of CD86 and PDL1 during the early stage of Scl GVHD. We demonstrated activation of splenic CD4$^+$ T cells in Scl GVHD that had increased numbers of CD44^{hi}CD62L^{neg} cells that produce IFNγ, IL-2, and TNFα, similar to what would be expected in the cytokine profile of Cyt GVHD (Ichiki *et al.*, 2006). In human scleroderma, both Th1- and Th2-like cytokines have been reported (Mavalia *et al.*, 1997; Valentini *et al.*, 2001; Abraham and Varga, 2005; Rottoli *et al.*, 2005). A caveat to these data on scleroderma patients is that when human scleroderma is diagnosed, it is often well established, so correlating cytokine production with triggers of disease may not be possible.

APC–T cell interactions in skin in Scl GVHD

APCs in the periphery (lymph node and spleen) are essential for activation of donor T cells in GVHD. On entry into the skin, these T cells will then encounter different populations of resident APCs. Cutaneous APCs have been shown to be essential for the development of Cyt GVHD, as replacement of recipient Langerhans cells (LC) with donor LC by low-dose T cell administration protected the recipient from developing GVHD on rechallenge with a large dose of T cells (Merad *et al.*, 2004). Neither cutaneous CD11c$^+$ DCs nor CD11b$^+$ macrophages freshly isolated from skin were capable of supporting proliferation of activated B10.D2 CD4$^+$ T cells, but they could support proliferation of activated C57BL/6 CD4$^+$ T cells. These data suggest that cutaneous APCs would not be supportive of T-cell proliferation in Scl GVHD.

The difference between normal function and pathology is often a fine balance between regulation of opposing factors. Although IFNγ and TNFα are thought to inhibit wound healing through their ability to suppress TGFβ signaling (Mori *et al.*, 2002; Ishida *et al.*, 2004), TGFβ can also inhibit IFNγ synthesis (Yu *et al.*, 2006). As we find mRNA for IFNγ and TGFβ but no TNFα in skin, the absence of TNFα may be, in part, responsible for pathology of Scl GVHD. Although we find some CD4$^+$ T cells that are capable of producing TNFα *ex vivo* on restimulation with anti-CD3, there were fewer than those found in control BMT (Figure 5). Our gene array data suggest that TNFα is not induced in skin during Scl GVHD at any time; these results reflect TNFα mRNA from both T cells and macrophages. Thus, TNFα, which has been shown to be essential for Cyt GVHD (Korngold *et al.*, 2003; Schmaltz *et al.*, 2003), is absent in the skin during Scl GVHD.

Therefore, we have identified three possible factors in the development of fibrosis rather than epithelial injury in Scl GVHD. First, cutaneous APCs, through their expression of the inhibitory molecule PDL1, do not support T-cell activation and no cytotoxic CD4$^+$ or CD8$^+$ T cells are generated. Second, the absence of TNFα-producing CD4$^+$ cutaneous T cells and TNFα mRNA in skin by gene array analysis during early Scl GVHD may be a key factor because TNFα induces keratinocyte apoptosis, leading to epithelial injury that is characteristic of Cyt GVHD. Third, TNFα is also a potent inhibitor of TGFβ-induced fibrosis (Yamane *et al.*, 2003; Schiller *et al.*, 2004). In some patients with Cyt GVHD, as TNFα levels in the serum increase, TGFβ levels decrease, suggesting a regulatory balance between these two cytokines (Remberger *et al.*, 2003; Visentainer *et al.*, 2003). There is some evidence that TGFβ production by T cells is important in regulating acute GVHD (Banovic *et al.*, 2005). In our model, we find splenic CD4$^+$ T cells that are capable of producing TNFα, but they are not found in skin. TNFα is elevated in the blood of patients with scleroderma compared with controls (Young *et al.*, 2002; Scala *et al.*, 2004), and activated peripheral blood mononuclear cell from these individuals also produce increased amounts of TNFα compared with controls (Scala *et al.*, 2004). However, these cells have not been demonstrated in the skin.

Implications for therapy in scleroderma

Our data suggest that the absence of TNFα production in skin may have profound effects on the development of Scl GVHD in its early stages. If the lack of TNFα promotes fibrosis, can TNFα induction be therapeutic for scleroderma and Scl GVHD? Th2-like CD4$^+$ T cells can inhibit collagen synthesis by dermal fibroblasts *in vitro* through the actions of membrane-bound TNFα (Chizzolini *et al.*, 2003). Thalidomide has been used in the treatment of scleroderma patients with some success (Browne *et al.*, 2000; Oliver *et al.*, 2000). Despite the ability of thalidomide to inhibit TNFα production by monocytes, this treatment result in the increase of serum levels of TNFα. This increase in serum TNFα would be due to increased production by other cell types including T cells. Therefore, selective targeting of skin to increase TNFα production may have some benefit in Scl GVHD.

A cautionary note in dealing with immunological disorders with complex interdependent pathways is that by inhibiting Scl GVHD, one may promote the development of Cyt GVHD. Continued testing of potential agents for scleroderma in mouse models is therefore critical to development of more effective therapies.

MATERIALS AND METHODS

BMT to generate Scl GVHD or Cyt GVHD

In a typical transplantation experiment, 5- to 8-week-old male and female B10.D2 (H-2^d) and Balb/c (H-2^d) mice (Jackson Laboratories, Bar Harbor, ME) are used as donors and recipients, respectively, for BMT to produce Scl GVHD (McCormick *et al.*, 1999; Zhang *et al.*, 2002a, 2003). C57BL/6 (H-2^b) and Balb/c (H-2^d) mice (Jackson laboratories) are used as donors and recipients, respectively, to generate Cyt GVHD. Briefly, female recipient mice are lethally irradiated with 700 cGy from a Gammacel 137Cs source (J.L. Shepard and Associates, San Fernando, CA). Mice are injected with male donor-BM cells (1.5×10^6/mouse) and spleen cells (3×10^6/mouse) suspended in RPMI-1640 (BioWhittaker, Walkersville, MD) with 20 U/ml heparin (Fisher Scientific, Pittsburgh, PA). A control group of female Balb/c mice receive male Balb/c BM and T cells (syngeneic BMT). We have used male donor cells and female recipient mice to track donor leukocytes in previous experiments (McCormick *et al.*, 1999; Zhang *et al.*, 2002a, 2003). Animals that do not engraft die within 10–14 days. Three to five animals are studied at each time point. Case Western Reserve University School of Medicine's Institutional Animal Care and Use Committee have approved all studies involving animals.

Tissue collection and RNA extraction, preparation, and characterization

At least three animals per group (experimental Scl GVHD and control BMT) per time point were killed via cervical dislocation 7, 14, 21, or 30 days post-BMT in six separate experiments. Back skin was chemically depilated and harvested for buffered formalin fixation (Surgipath Medical Industries, Richmond, IL), paraffin embedding (Sakura Finetech, Torrance, CA), routine histology for hematoxylin and eosin staining and for measurement of skin thickness (McCormick *et al.*, 1999; Zhang *et al.*, 2002a, 2003). Skin was also collected for flow cytometric analysis and for RNA extraction (snap-frozen in liquid nitrogen and stored at −80°C). Total RNA was isolated using TRIzol reagent as per manufacturer's protocol (GibcoBRL, Carlsbad, CA). RNA quality was assessed by spectrophotometer at 260/280 nm absorption and the integrity and overall quality was evaluated by native agarose gel electrophoresis. Biotinylated riboprobes synthesized from total RNA specimens were initially hybridized to an Affymetrix Test3 miniarray (Affymetrix, Santa Clara, CA) to verify probe quality.

Gene expression analysis

Gene expression was interrogated using Affimetrix Mouse Genome Arrays 430A, which contain 22,600 oligonucleotide probe sets for approximately 14,000 well-characterized genes. Total skin RNA was used to prepare biotinylated target cRNA, with minor modifications from the manufacturer's recommendations. The target biotinylated cRNA was processed as per manufacturer's recommendation using an Affymetrix GeneChip instrument system. Arrays were then washed and stained with steptavidin–phycoerythrin before being scanned on an Affymetrix GeneChip scanner. The fluorescent intensity of each probe was quantified using Microarray Analysis Suite version 5.0 (Affymetrix). Additional annotation data was incorporated into the data set using the Affymetrix web-based analysis tool NetAffx. The signals displayed for the genes in each sample that were included in the trimmed data set were imported into GeneSpring software version 7.2 (Silicon Genetics, San Carlos, CA). To present the relative expression for a given gene or probe set in each sample, the measured signal for each probe set was normalized to sample 1, which was from normal female Balb/c mouse skin. One-way hierarchical clustering with Pearson correlation analyses and minimum distance of 0.001 was employed to order genes in the trimmed data set for the time course of the two groups. Details of gene array analysis on murine Scl GVHD are published separately (Zhou *et al.*, 2007).

Preparation of single-cell suspensions

Spleen was minced into small pieces and digested in RPMI-5% fetal bovine serum (Hyclone, Logan, UT) containing 300 U/ml collagenase type 3 (Worthington Biochemical, Lakewood, NJ) and 30 U/ml DNase I (Sigma-Aldrich, St Louis, MO) at 37°C for 1 hour. Depilated skin was digested in RPMI-5% fetal bovine serum containing 500 U/ml Collagenase type 3, 30 U/ml DNase I and 1000 U/ml Hyaluronidase (Worthington) at 37°C for 1 hour. Cells were then passed through a 70-μm cell strainer Falcon (Bectin Dickenson Labware, Franklin Lakes, NJ) to generate single-cell suspensions. Dead cells and cellular debris were removed from the single-cell suspensions by placing cell suspensions on a discontinuous gradient with Ficoll-Paque (Pharmacia, Uppsala, Sweden).

Flow cytometry of single-cell suspensions

Antibodies and appropriate isotype controls (eBioscience, San Diego, CA or BD Pharmingen, San Diego, CA) were used as follows: anti-CD4 (clone RM4-5), anti-CD11b (clone M1/70), anti-CD11c (clone N418), anti-CD44 (clone IM7), anti-CD62L (clone MEL-14), anti-CD86 (clone GL1), anti-PDL1/B7-H1 (MIH5:), anti-IFNγ (clone XMG1.2), anti-IL-2 (clone JES6-5H4), and anti-TNFα (clone MP6-XT22). Isolated cells were incubated for 20 minutes at 4°C in blocking buffer which contained 10% normal mouse serum in FACS buffer (0.1% bovine serum albumin in phosphate-buffered saline). Cells were washed twice in FACS buffer and fixed using Cytofix/perm solution (BD Pharmingen). For intracellular cytokine staining, single-cell suspensions from spleen or skin were incubated on anti-CD3-coated plates for 5–6 hours in RPMI-5% fetal bovine serum containing 1 μg/ml brefeldin A (Golgiplug, BD Pharmingen). Cells were then incubated with blocking buffer for 20 minutes at 4°C, followed by incubation with anti-CD4 for an additional 20 minutes. Cells were then fixed with paraformaldehyde, washed in the presence of saponin (Perm/Wash buffer, BD Pharmingen), incubated with fluorescent anti-cytokine antibodies, washed, and examined on a LSR I flow cytometer (BD Bioscience). All data were analyzed using FlowJo analysis software (Tree Star Inc., Ashland, OR).

Purification of APCs and T cells by magnetic bead isolation

$CD11c^+$ DCs and $CD11b^+$ macrophages were isolated from Balb/c spleen and skin with anti-$CD11c^+$- and anti-$CD11b^+$-coated magnetic beads using positive selection (Miltenyi Biotec Inc.,

Auburn, CA). Single-cell suspensions from tissue were incubated first with anti-CD11c$^+$-coated microbeads and then separated on magnetic columns. The flow through, which comprised CD11c$^-$ cells, was then incubated with anti-CD11b$^+$-coated microbeads and isolated on magnetic columns. By this method we were able to separate CD11b$^+$ DCs from CD11b$^+$ macrophages. CD4$^+$ T cells were enriched from B10.D2 mouse tissue by negative selection using CD4 T cell isolation kits respectively (Miltenyi Biotec Inc.). Approximately 1×10^5 CD11c$^+$ DCs and 5×10^5 CD11b$^+$ macrophages could be isolated from 0.5 g of skin. Purity of cells was determined by flow cytometry for each population (>80%).

Mixed leukocyte cultures

Cultures were established using CD11b$^+$ macrophages or CD11c$^+$ DCs isolated from either skin or spleen of Balb/c mice as stimulatory APCs and responding CD4$^+$ T cells isolated from spleen of B10.D2 mice. For primary proliferation cultures, APCs were added at different concentrations in the presence of 2×10^5 CD4$^+$ T cells in 96-well plates (Falcon). Cultures were incubated at 37°C for 4 days. For secondary proliferation CD4$^+$ T cells were first cultured with CD11c$^+$ splenic DCs for 4 days. CD4$^+$ T cells were purified from cultures through negative selection using CD11c$^+$ microbeads. CD4$^+$ T cells were then cultured for an additional 4 days in the presence of media alone, CD11c$^+$ splenic DCs, CD11c$^+$ cutaneous DCs, or CD11b$^+$ cutaneous macrophages. For proliferation studies, 1.0 μCi of ^{3}H-thymidine (Perkin Elmer Life Science, Boston, MA) was added to the culture for the last 8 hours. Cells were then harvested and the amount of ^{3}H-thymidine incorporated into T cells was measured. For ELISA studies, supernatant was collected from primary and secondary cultures at specific time points and frozen at −80°C. Supernatant was then analyzed using Quantikine kit for TGFβ (R&D systems, Minneapolis, MN). For ELISA experiments, RPMI containing 5% normal mouse serum (Jackson ImmunoResearch, West Grove, PA) was used in place of RPMI-5% fetal bovine serum.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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